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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

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24741-1526

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

INTERNATIONAL APPLICATION NO. PCT/EP99/09444	INTERNATIONAL FILING DATE 03 December 1999 (03.12.99)	U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.52) 097857343 PRIORITY DATE CLAIMED 03 December 1998 (03.12.98)
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TITLE OF INVENTION

POROUS COMPOSITE MATRIX AND THE PRODUCTION AND USE THEREOF

APPLICANT(S) FOR DO/EO/US


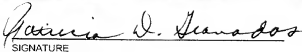
Peter ANGELE and Richard KUJAT

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371©(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371 ©(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371©(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371©(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371©(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371©(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - (i) Copy of English language Search Report (Form PCT/ISA/210)

U.S. APPLICATION NO. 09/1857343 INTERNATIONAL APPLICATION NO. PCT/EP99/09444		ATTORNEY'S DOCKET NUMBER 24741-1526	
17. <input type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY	
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... \$690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... \$100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than 20 ____ 30 ____ months from the earliest claimed priority date (37 CFR 1.492(e))		\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	97 -20 =		X \$18.00 \$
Independent Claims	8 -3 =		X \$80.00 \$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00 \$
TOTAL OF ABOVE CALCULATIONS =		\$860.00	
Applicants claim small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		\$	
SUBTOTAL =		\$860.00	
Processing fee of \$130.00 for furnishing English translation later than 20 ____ 30 ____ months from the earliest claimed priority date (37 CFR 1.492(f)).		\$	
TOTAL NATIONAL FEE =		\$860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +		\$	
TOTAL FEES ENCLOSED =		\$860.00	
		Amount to be:	
		refunded \$	
		charged: \$	
a. <input checked="" type="checkbox"/> A check in the amount of \$860.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 08-1641 in the amount of \$ ____ to the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-1641 . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO: Customer ID No. 26633 Patricia D. Granados HELLER EHRMAN WHITE & MCAULIFFE, LLP 1666 K Street, NW, Suite 300 Washington, DC 20006 Tel: (202) 912-2000 Fax: (202) 912-2020			
			
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		REGISTRATION NUMBER: 33,683	
		DATE: JUNE 4, 2001	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

09/857343

In re patent application of:
Peter ANGELE et al.

Application No.: 09/857,3431

Art Unit: Unassigned

Filed: June 4, 2001

Examiner: Unassigned

For: POROUS COMPOSITE MATRIX AND THE PRODUCTION AND USE THEREOF

Preliminary Amendment

Commissioner for Patents
Washington, DC 20231
Box PCT

Sir:

Before action by the Examiner, Applicants respectfully request entry of the Amendments set forth in the Amendments below and in the attached Marked Copy. Applicants believe that no fee is required. If this is incorrect, the Director is hereby authorized to credit any overpayment or to charge any deficiency to Deposit Account 08-1641.

Amendments

Please cancel claims 20-22 without prejudice or disclaimer and amend the remaining claims as follows:

1. (Amended) A porous composite matrix, comprising a matrix constructed from matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and wherein the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1.

2. (Amended) The composite matrix as claimed in claim 1, wherein the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 60:40 to 99:1.

3. (Amended) A composite matrix as claimed in claim 1, wherein the hydrolyzed collagen is partially or completely hydrolyzed.

4. (Amended) A composite matrix as claimed in claim 1, wherein the hydrolyzed collagen is additionally derivatized and/or crosslinked.

5. (Amended) A composite matrix as claimed in claim 1, wherein the hyaluronic acid derivative is a hyaluronic acid ester.

6. (Amended) A composite matrix as claimed in claim 5, wherein the hyaluronic acid ester is an ethyl or benzyl ester of hyaluronic acid.

7. (Amended) A composite matrix as claimed in claim 1, further comprising pores having an average diameter in the range of 10-1000 μm .

8. (Amended) A composite matrix as claimed in claim 7, wherein the pores have an average diameter in the range of 100-350 μm .

9. (Amended) A composite matrix as claimed in claim 7, wherein the pores have an average diameter in the range of 350-1000 μm .

10. (Amended) A composite matrix as claimed in claim 8 further comprising pores in the range of 10-100 μm .

11. (Amended) A composite matrix as claimed in claim 1, further comprising crosslinkages.

12. (Amended) A composite matrix as claimed in claim 1, further comprising biologically active compounds.

13. (Amended) A composite matrix as claimed claim 1, further comprising chondrocytes, mesenchymal stem cells, mesenchymal progenitor cells, osteoblasts and connective tissue cells.

14. (Amended) A process for the production of a porous composite matrix as claimed in claim 1, comprising:

dissolving or suspending a hyaluronic acid derivative and a hydrolyzed collagen in a suitable first solvent to form a solution or suspension,

adding a pulverulent compound to the solution or suspension, wherein the pulverulent compound is virtually insoluble in the first solvent, and wherein the pulverulent compound is soluble in a second solvent, in which the matrix formers hyaluronic acid derivative and hydrolyzed collagen are virtually insoluble, wherein the pulverulent compound has an average particle size distribution in the range of the desired pore size of the composite matrix to be produced,

removing the first solvent, and

dissolving the pulverulent compound in the second solvent, in which the pulverulent compound dissolves and the matrix formers are virtually insoluble to obtain said porous composite matrix.

15. (Amended) The process as claimed in claim 14, wherein the first solvent is 1,1,1,3,3,3-hexafluoro-isopropanol.

16. (Amended) The process as claimed in claim 14, wherein the pulverulent compound is a water-soluble alkali metal or alkaline earth metal salt.

17. (Amended) The process as claimed in claim 14, wherein the second solvent is water.

18. (Amended) The process as claimed in claim 14, wherein the composite matrix is additionally shaped, dried and optionally sterilized.

19. (Amended) The process as claimed in claim 14, wherein the composite matrix is additionally loaded with biologically active compounds and chondrocytes, mesenchymal stem and progenitor cells, osteoblasts or connective tissue cells.

23. (Amended) An implant, comprising a porous composite matrix as claimed in claim 1.

24. (Amended) A process for the production of an implant as claimed in claim 23, comprising coating a porous composite matrix onto a surface of said implant, wherein said porous composite matrix comprises a matrix constructed from matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and wherein the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1.

Please add the following new claims:

25. (New) The composite matrix as claimed in claim 1, wherein the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of approximately 70:30.

26. (New) A composite matrix as claimed in claim 9, further comprising pores in the range of 10-100 μm .

27. (New) The process as claimed in claim 14, wherein the pulverulent compound is an alkali metal halide.

28. (New) The process as claimed in claim 14, wherein the pulverulent compound is sodium chloride.

29. (New) The process as claimed in claim 15, wherein the pulverulent compound is a water-soluble alkali metal or alkaline earth metal salt.

30. (New) The process as claimed in claim 15, wherein the pulverulent compound is an alkali metal halide.

31. (New) The process as claimed in claim 15, wherein the pulverulent compound is sodium chloride

32. (New) A process for generating differentiated tissue from chondrocytic cells or mesenchymal stem and progenitor cells, comprising adding freshly removed or amplified cells to a composite matrix as claimed in claim 1, and optionally culturing said cells under chondro-, osteo- or fibrogenic conditions.

33. (New) A process for generating differentiated tissue from chondrocytic cells or mesenchymal stem and progenitor cells, comprising culturing cells added to a composite matrix as claimed in claim 1 under chondro-, osteo- or fibrogenic conditions.

34. (New) The process as claimed in claim 32, wherein said process is for the tissue engineering of tissue types of the connective and supportive apparatus.

35. (New) The process as claimed in claim 32, wherein said process is for the in-vivo differentiation of the cells to tissue types of the connective and supportive apparatus.

36. (New) The process as claimed in claim 34, wherein said tissue is chondral and osseous tissue.

37. (New) The composite as claimed in claim 12, wherein the biologically active compounds are selected from the group consisting of antibiotics, compounds for improving cell adhesion, calcium salts, inductive factors, and further glycosaminoglycans and their derivatives.

38. (New) The process as claimed in claim 35, wherein said tissue is chondral or osseous tissue.

Remarks

By the foregoing, claims 1-19, 23 and 24 have been amended, claims 20-22 have been canceled, and claims 25-38 have been added. Accordingly, claims 1-19, 23-38 will be pending with entry of this amendment. Support for the amendments and new claims are found throughout the originally filed specification and claims, an English copy of which is filed herewith.

An action on the merits is now awaited. Should there be any questions the Examiner is invited to contact the undersigned at the local exchange listed below.

Respectfully submitted,

Sept. 4, 2001
Date

for *Patricia I. Granados*
Patricia I. Granados
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PATENT TRADEMARK OFFICE

Marked Copy

Please cancel claims 20-22 without prejudice or disclaimer and amend the remaining claims as follows:

1. (Amended) A porous composite matrix, **[in which the] comprising a** matrix **[is]** constructed from matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and **wherein** the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1.

2. (Amended) The composite matrix as claimed in claim 1, **[in which] wherein** the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 60:40 to 99:1**[, preferably in a weight ratio of approximately 70:30]**.

3. (Amended) A composite matrix as claimed in **[one of the preceding claims] claim 1, [in which] wherein** the hydrolyzed collagen is partially **[and/]or** completely hydrolyzed.

4. (Amended) A composite matrix as claimed in **[one of the preceding claims] claim 1, [in which] wherein** the hydrolyzed collagen is additionally derivatized and/or crosslinked.

5. (Amended) A composite matrix as claimed in **[one of the preceding claims] claim 1, [in which] wherein** the hyaluronic acid derivative is a hyaluronic acid ester.

6. (Amended) A composite matrix as claimed in claim 5, **[in which] wherein** the hyaluronic acid ester is an ethyl or benzyl ester of hyaluronic acid.

7. (Amended) A composite matrix as claimed in **[one of the preceding claims], claim 1, further** comprising pores having an average diameter in the range of 10-1000 µm.

8. (Amended) A composite matrix as claimed in claim 7, **[in which] wherein** the pores have an average diameter in the range of 100-350 µm.

9. (Amended) A composite matrix as claimed in claim 7, **in which wherein** the pores have an average diameter in the range of 350-1000 μm .

10. (Amended) A composite matrix as claimed in claim 8, **for 9, in which further comprising** pores in the range of 10-100 μm **are additionally present**.

11. (Amended) A composite matrix as claimed in **one of the preceding claims, which has claim 1, further comprising** crosslinkages.

12. (Amended) A composite matrix as claimed in **one of the preceding claims, claim 1, further** comprising biologically active compounds **such as antibiotics, compounds for improving cell adhesion, calcium salts, inductive factors or further glycosaminoglycans and their derivatives**.

13. (Amended) A composite matrix as claimed **in one of the preceding claims, claim 1, further** comprising chondrocytes, mesenchymal stem **cells, and mesenchymal** progenitor cells, osteoblasts **or and** connective tissue cells.

14. (Amended) A process for the production of a porous composite matrix as claimed in **one of claims 1-13 claim 1,** comprising:

[the dissolution or suspension of the] dissolving or suspending a hyaluronic acid derivative and **[the] a** hydrolyzed collagen in a suitable first solvent **to form a solution or suspension,**

[the addition of] adding a pulverulent compound **to the solution or suspension, wherein the pulverulent compound [which] is virtually insoluble in the first solvent, and wherein the pulverulent compound [but which] is soluble in a second solvent, in which the matrix formers hyaluronic acid derivative and hydrolyzed collagen are virtually insoluble, [to the solution or suspension], wherein the pulverulent compound [having] has** an average particle size distribution in the range of the desired pore size of the composite matrix to be produced,

[the removal of] removing the first solvent, and

[subsequently the dissolution of] dissolving the pulverulent compound in [a] the second solvent, in which the pulverulent compound dissolves and the matrix formers are virtually insoluble to obtain said porous composite matrix.

15. (Amended) The process as claimed in claim 14, [in which] wherein the first solvent is 1,1,1,3,3,3-hexafluoro-isopropanol.

16. (Amended) The process as claimed in claim 14, [or 15, in which] wherein the pulverulent compound is a water-soluble alkali metal or alkaline earth metal salt[, in particular an alkali metal halide such as sodium chloride].

17. (Amended) The process as claimed in [one of claims 14-16] claim 14, [in which] wherein the second solvent is water.

18. (Amended) The process as claimed in [one of claims 14-17] claim 14, [in which] wherein the composite matrix is additionally shaped, dried and optionally sterilized.

19. (Amended) The process as claimed in [one of claims 14-18] claim 14, [in which] wherein the composite matrix is additionally [optionally] loaded with biologically active compounds and chondrocytes, mesenchymal stem and progenitor cells, osteoblasts or connective tissue cells.

23. (Amended) An implant, comprising a porous composite matrix as claimed in [one of claims 1-13] claim 1.

24. (Amended) A process for the production of an implant as claimed in claim 23, comprising coating a porous composite matrix onto a surface of said implant, wherein said porous composite matrix comprises a matrix constructed from matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and wherein the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1 [in which a porous composite matrix as claimed in one of claims 1-13 is coated onto the implant surface].

Porous composite matrix, its production and use

5

The invention relates to a porous composite matrix formed from a hyaluronic acid derivative and hydrolyzed collagen, which is used as a biocompatible and biodegradable composite matrix for the repair of musculoskeletal defects.

10

For the regeneration of tissue defects, it is necessary to use a body-compatible, slowly biodegradable matrix which under suitable conditions makes possible the differentiation of introduced cells with marked production of a specific intercellular matrix. Various matrices of this type are known in the prior art.

15

WO 97/28192 discloses a process for the preparation of prion-free collagen products which can be used as a spongy implant. For ocular applications, the collagen product can be crosslinked with 5% by weight hyaluronic acid to increase the transparency. The hyaluronic acid additionally stimulates the cell infiltration into the implant.

20

WO 91/18558 and WO 91/16867 and also US 4,880,429 disclose composite matrices formed from collagen and up

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WO 91/18558 and WO 91/16867 and also US 4,880,429 disclose composite matrices formed from collagen and up to 25% by weight of glycosaminoglycans such as hyaluronic acid.

5

EP-A-0 784 985 discloses a porous composite article which comprises a bioabsorbable hydrophilic material selected from gelatin, hyaluronic acid and a hyaluronic acid derivative. To avoid premature absorption, the article is additionally provided with a polymer layer which is absorbed in a delayed manner.

10

WO 97/14376 discloses a bone transplant matrix formed from collagen, which can contain hyaluronic acid as a binding agent.

15

US 5,676,964 discloses inter- and intramolecularly crosslinked esters of acidic polysaccharides such as preferably hyaluronic acid. These esters can be employed as biodegradable, for example spongy, materials as surgical articles.

20

The known matrices, however, show marked restrictions in the provision of suitable medium conditions for the differentiation of introduced cells (e.g. premature absorption, unsuitable matrix composition) and are also unsatisfactory with respect to the stability necessary for handling.

25

An object of the present invention thus consists in making available a matrix which assists cell differentiation and intercellular matrix production and is then itself slowly degraded. Moreover, the matrix should have an adequate stability which makes the matrix highly suitable and easy to handle not only for preculture of cells in vitro but also for in-vivo implantation.

30

35

It has now been found that this object is achieved by a composite matrix formed from a hyaluronic acid derivative and hydrolyzed collagen of specific composition.

5

The present invention thus relates to a porous composite matrix, the matrix being synthesized on matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and the matrix formers being present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1.

10

As matrix former, the composite matrix according to the invention comprises a hyaluronic acid derivative and hydrolyzed collagen, preferably in a weight ratio of 60:40 to 99:1 and particularly preferably of approximately 70:30.

15

Preferably, the matrix is synthesized only from the hyaluronic acid derivative and the hydrolyzed collagen.

20

It has been shown that matrices having a proportion of hyaluronic acid derivative of below 30% by weight are technically disadvantageous on account of too low a stability. Conversely, it was possible by means of the composite matrix according to the invention comprising a hyaluronic acid derivative and hydrolyzed collagen markedly to improve cell adhesion, matrix loading with cells and subsequent cell differentiation compared with matrices comprising 100% hyaluronic acid derivative, such as are known, for example, from US 5,676,964 mentioned at the outset.

25

30

Moreover, a component which can be degraded in a delayed manner is absorbed directly into the composite matrix by the hyaluronic acid derivative. By means of this, for example, the additional coating disclosed in EP-A-0 784 985 can be avoided, or a collagen derivatization which may otherwise be necessary, which

35

is undesirable on account of the toxicity of the derivatizing agent which has been detected in some cases.

5 A suitable hydrolyzed collagen is partially and/or completely hydrolyzed collagen, in particular gelatin, i.e. collagen in highly hydrolyzed form. For example, gelatin from pigs or cattle can be employed. However, forms of gelatin having a higher aggregation rate along
10 the lines of fibrils can also be employed. More highly aggregated collagen can lead to an improvement in matrix stability. Those collagens having degradation forms of different size can be produced by a controlled, slow hydrolysis of fibrillar collagen.

15 If desired, the hydrolyzed collagen can additionally be derivatized and/or crosslinked.

20 As a hyaluronic acid derivative for the preparation of the composite matrix according to the invention, hyaluronic acid esters such as ethyl esters and in particular benzyl esters are preferred on account of their better biomechanical properties, it being possible for the hyaluronic acid to have different
25 degrees of esterification. Examples of hyaluronic acid esters which can be employed according to the invention are mentioned in US patent No. 5,676,964. The disclosure of this US patent is thus incorporated into the present description.

30 Advantageously, the hyaluronic acid derivative employed is predominantly hydrophobic.

35 A preferred hyaluronic acid ester is a benzyl ester of hyaluronic acid (HYAFF), which can be ordered, for example, from "Fidia Advanced Biopolymers" from Abano Terme in Italy. HYAFF is supplied in various degrees of esterification, of which a highly esterified benzyl hyaluronate "HYAFF 11" (100% benzyl ester), which is

commercially obtainable as the already licensed wound dressing material "JALOSKIN", is preferred according to the invention.

- 5 Other hyaluronic acid esters having lower degrees of esterification (for example HYAFF 11 p 75, a benzyl hyaluronate having a degree of esterification of about 75%) and/or other alcohol radicals, such as ethyl hyaluronate (for example HYAFF 7), or with mixtures of
10 various alcohol radicals, however, can also be employed.

- The composite matrix according to the invention is porous, in particular open-pore. Preferably, the pores
15 in the composite matrix have an average diameter in the range of 10-1000 μm , in particular 50-500 μm . It has been shown that excessively large pores ($> 1000 \mu\text{m}$) lead on population with cells to a high cell loss from the matrix, particularly with small sponge diameters.
20 With excessively small pores ($> 100 \mu\text{m}$), a strong sieve effect is seen and the cells cannot be populated in deeper regions of the matrix. However, a lower density and a looser structure of the composite matrix can be achieved by means of a specific proportion of smaller
25 pores. By this means, an acceleration of the degradation in vivo can be achieved without alteration of the pore size accessible to the cells.

- Pores having an average diameter in the range of 100-
30 350 μm and pores having an average diameter in the range of 350-1000 μm have proven advantageous. If a lower density or a looser structure of the composite matrix is desired, pores in the range of 10-100 μm , in particular in the range of approximately 50 μm , can
35 additionally be present. Pores of approximately equal size or pores having a size gradient can also be made available.

The composite matrix according to the invention can additionally be chemically or physically crosslinked. By this means, the biodegradability of the composite matrix can be delayed if required. Moreover, premature
5 leaching of possible additives can be prevented. A suitable crosslinking agent is, for example, cyanamide, which crosslinks proteins and polysaccharides and on biological degradation produces no exogenous, harmful residues, as it is degraded to urea.

10 The composite matrix according to the invention can moreover comprise biologically active compounds. These can be, for example, compounds which optimize the property of the matrix of populating cells, for example
15 antibiotics, compounds for improving cell adhesion, calcium salts, inductive factors or further glycosaminoglycans and their derivatives. Advantageously, cell adhesion can be improved by addition of highly polymeric poly-L-lysine or coating
20 with an activated succinyl derivative of poly-L-lysine or the admixture of fibronectin or peptides having RGD sequences.

25 In order to optimize the use of the composite matrix according to the invention in the therapy of osseous tissue defects, the matrix can contain calcium salts such as, for example, calcium sulfates, calcium phosphates and calcium carbonates, for example as suspension or solution.

30 To decrease the risk of infection on the implantation of the composite matrix according to the invention, said matrix can also contain antibiotics.

35 As further biologically active compounds, the composite matrix according to the invention can contain, for example, inductive factors for optimizing the repair of musculoskeletal defects, in particular cytokines such

as bFGF (fibroblast growth factor), IGF (insulin-like growth factor) or TGFbeta (transforming growth factor).

The composite matrix is particularly suitable for the in-vitro and in-vivo generation of differentiated tissue from chondrocytic cells, mesenchymal stem and progenitor cells, osteoblasts and connective tissue cells. The invention thus also relates to composite matrices which comprise these cells.

A further aspect of the present invention is a process for the production of the porous composite matrix described above. This process comprises dissolving or suspending the hyaluronic acid derivative and the hydrolyzed collagen in a suitable first solvent, the addition of a pulverulent compound which is virtually insoluble in the first solvent, but which is soluble in a second solvent, in which the matrix formers hyaluronic acid derivative and hydrolyzed collagen are virtually insoluble, to the solution or suspension, the pulverulent compound having an average particle size distribution in the range of the desired pore size of the composite matrix to be produced, the removal of the first solvent and subsequently the dissolution of the pulverulent compound in a second solvent, in which the pulverulent compound dissolves and the matrix formers are virtually insoluble.

A suitable first solvent is, in particular, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). This is a highly volatile liquid in which esterified hyaluronic acid, hydrolyzed collagen (gelatin) and further substances necessary for the specific requirements, such as growth factors and calcium compounds, can be simultaneously dissolved or suspended at room temperature. The larger the molecular units of the hydrolyzed collagen, the poorer their solubility in HFIP. Fibrillar collagen is no longer dissolved.

The concentrations of the starting substances in the first solvent are insignificant for the process according to the invention and can be varied as long as handleable solutions or suspensions are obtained. This relates both to the concentrations of the individual components, and the total concentration of the components in the first solvent. The individual concentrations of the hyaluronic acid derivative and the hydrolyzed collagen determine the weight ratio of the two components in the final product. Finally, as the first solvent is stripped off the final product, the total concentration determines the density and solidity of the final product.

For the handling of the composite matrix according to the invention, its mechanical strength in the wet state plays an important role. The solidity of the composite matrix is greatest if there is a high hyaluronic acid derivative content in the matrix, as the matrix swells the least here. With growing content of the hydrolyzed collagen, the composite matrix swells increasingly strongly and becomes less stable.

Preferably, 5% by weight HYAFF is dissolved in HFIP in the process according to the invention. For this, gelatin can be admixed in a variable individual concentration of, for example, up to 7.5% by weight, such that a high total concentration of 12.5% by weight of matrix former is achieved in the solution. On account of this, the decrease in the solidity is compensated because of the strong swelling. Solutions having a total concentration of more than 12.5% by weight are very viscous and are difficult to handle. An increase in the content of gelatin in the final product can therefore only be carried out if the HYAFF content is reduced. However, a reduction in the HYAFF content (for example to an individual concentration of 2.77% by weight with a total concentration of 9.23% by weight of the matrix former in the solution, which corresponds to

a weight ratio of HYAFF to gelatin of 30:70) decreases the stability of the matrix without producing an improvement in the biocompatibility.

5 In principle, the addition of gelatin has a positive effect on the biocompatibility and on the histogenic property of the matrix. A weight ratio of hyaluronic acid derivative to hydrolyzed collagen of approximately 70:30 proved suitable in vitro and in vivo, but,
10 depending on the application, for example for the formation of cartilage or for the regeneration of bone tissue, optimum weight ratios can be in the range from 99:1 to 60:40.

15 Pore formation is carried out by addition of a pulverulent compound which is virtually insoluble in the first solvent. If HFIP is used as the first solvent, sodium chloride, for example, is suitable as a pulverulent compound for pore formation. Sodium
20 chloride is virtually insoluble in HFIP, moreover it is nontoxic and cheap.

In addition to sodium chloride, any alkali metal or alkaline earth metal salt, in particular halide, which
25 is water-soluble and insoluble in HFIP is suitable as the first solvent when using HFIP. For the reasons mentioned above, however, sodium chloride is preferred.

The amount of the pulverulent compound added determines
30 the pore number and thus the density and also solidity of the matrix produced. The weight ratio of solution or suspension to sodium chloride crystals of approximately 1:2 has proven to be advantageous.

35 The pore size is determined by the selection of the particle size of the pulverulent compound. Commercially available sodium chloride mainly has particles having a diameter of between 500 and 1000 μm . Fractions of smaller size can easily be produced by grinding larger

grains in a mortar and by screening through calibrated screens.

The mixture of HYAFF, gelatin and sodium chloride crystals has the consistency of a thick paste. By pressing with a die in molds, for example of inert plastic (PTFE, PE, PVC), it is possible to produce matrix objects whose shape can largely be adapted to requirements. As HFIP is very volatile as a solvent, rapid drying of the mixture takes place. Because of this, the mixture should be stored in closed vessels and processed as quickly as possible. Drying can take place, for example, overnight under a hood and subsequently for a few hours in vacuo. The composite matrix can then be removed from the mold.

As the mixture scarcely shrinks during drying, releasing the matrix can cause difficulties. Because of this, the molds should be designed such that the dried contents can be forced out with a ram. For example, cylindrical matrices having a diameter of 3-18 mm and a height of 2-15 mm can easily be produced. For the production of larger matrix blocks, cuboid, trough-shaped molds which are put together from dismantlable wall and bottom parts have proven particularly suitable.

The pores of the composite matrix according to the invention are then obtained by dissolving the pulverulent compound in a second solvent in which the pulverulent compound dissolves and the matrix formers are virtually insoluble. If sodium chloride crystals are used as pulverulent compound, a suitable second solvent is in particular water. Repeated rinsing in pure water removes the salt and possibly still adherent traces of HFIP. In the case of small samples, four changes of the second solvent are recommended after an immersion time of 15 minutes in each case, with larger samples 6 changes after 20 minutes in each case.

On the first drying of the composite matrix by evaporating the first solvent, closed pores having grains of salt contained therein are primarily produced. During the washing process, the semi-permeable substance of the matrix swells and the formation of an osmotically highly active salt solution occurs in the pores. As a result of this, on further absorption of water the pores burst and the matrix finally becomes open-pore.

If desired, the composite matrix can additionally be loaded with biologically active compounds as mentioned above by way of example during or after production. Advantageously, the biologically active compounds are added to the solution or suspension of the matrix components even before the addition of the pulverulent compound.

The composite matrix thus produced is advantageously then dried. This can be achieved, for example, by immersion in acetone of increasing concentration (50%, 80%, 100%), blotting on filter paper and subsequent drying in vacuo.

Finally, it is advisable to remove a thin surface layer of the composite matrix, for example using a sharp blade, as a high number of closed pores remains in this layer, which prevents the deep penetration of cells.

Before loading with cells, the composite matrix is advantageously sterilized. This can be carried out by various known sterilization processes such as with alcohol, ethylene oxide or by gamma sterilization. A gamma sterilization with, for example, 350,000 rad is preferred.

The composite matrix according to the invention is suitable for the in-vitro and in-vivo generation of

differentiated tissue from chondrocytic cells, mesenchymal stem and progenitor cells, osteoblasts and connective tissue cells. On account of a specifically adapted matrix composition and matrix geometry, the repair of musculoskeletal defects is hereby possible.

The present invention thus also relates to the use of the composite matrix described above for the generation of differentiated tissue from chondrocytic cells or mesenchymal stem and progenitor cells, in which freshly removed or amplified cells are added to the composite matrix and optionally cultured under chondro-, osteo- or fibrogenic conditions.

By addition of freshly removed chondrocytes or mesenchymal stem and progenitor cells or of dedifferentiated chondrocytes or mesenchymal stem and progenitor cells which are amplified in vitro to the composite matrix according to the invention under chondrogenic culture conditions, a biomechanically loadable joint cartilage tissue can thus be prepared. The composite matrix according to the invention is thus suitable for the tissue engineering of tissue types of the connective and supportive apparatus, in particular of chondral and osseous tissue.

A biocompatible and biodegradable composite matrix produced in this way is thus suitable without or with prior in-vitro culturing for in-vivo differentiation to give tissue types of the connective and supportive apparatus, in particular to give chondral and osseous tissue under ectopic or autotopic implantation.

The composite matrix according to the invention is suitable, for example, for human chondrocytes which have been obtained from hyaline cartilage. Hyaline joint cartilage from autopsies and residual cartilage supplies from the carrying-out of total endoprostheses can be the origin of removal here. Embryonic

chondrocytes, such as can be obtained, for example, from abortions are additionally suitable. Moreover, adult mesenchymal stem and progenitor cells, for example from bone marrow, synovium or periostium and preferably embryonic mesenchymal stem and progenitor cells, for example from the umbilical cord, can be used. Embryonic mesenchymal stem cells offer the advantage of a lack of rejection in allogenic transplantation. Moreover, they are constantly available in adequate number and can be obtained without an additional intervention on the patient.

A further aspect of the present invention is an implant which comprises a porous composite matrix according to the invention. Such an implant, which is preferably coated with the composite matrix, has the advantage that better integration in the bone and optionally also the surrounding connective tissue is produced.

Suitable implant surfaces which are coated with the composite matrix according to the invention are, in particular, metal surfaces, such as titanium or steel, a polymer or ceramics.

The composite matrix according to the invention has the advantage that, on loading the matrix with cells, only a small change in size occurs. On loading with cells, known collagen matrices show great differences in size (initially massive swelling, then a strong tendency to form into a ball). For use of tissue engineering (in-vitro production of tissue with the aim of incorporating this into a defect), however, size stability of the matrix is also of great importance after cell loading and culturing. This is achieved by the composite matrix according to the invention and in particular by the simultaneous mixing of the matrix formers using HFIP.

Moreover, the following further advantages are achieved using the composite matrix according to the invention:

5 In stationary culture, a redifferentiation of amplified chondrocytes in the composite matrix is possible. Cartilage-typical proteoglycans (chondroitin sulfate, keratan sulfate, aggrecan) and collagen II are formed. It was possible to detect these after culturing for 2, 4 and 6 weeks.

10

The product produced in vitro from chondrocytes cultured on the composite matrix shows a marked increase in biomechanical stability in comparison to the starting condition at the point in time of the application of cells to the matrix.

15

Without cells, the matrix dissolves in vitro after about 14 days. In vivo, resorption can be observed after about 6-8 weeks (nude mice, rabbits).

20

Good differentiation ability of bone marrow cells to hyaline-like tissue in vitro.

25

Very good differentiation to give osseous tissue on incorporation into subcutaneous tissue of nude mice, resorption of the matrix in vivo only after 6 weeks.

30

On incorporation into osteochondral defects in the knee joint of rabbits, integration of the cell matrix construct is recognizable (cells were in this case bone marrow cells or chondrocytes), differentiation to cartilaginous tissue is recognizable. In the osseous defect portion, bone integration and differentiation to give new bone tissue is seen.

35

No proneness to inflammation by material in the knee joint detectable (no hyarthrosis, no massive increase in inflammatory cells).

Incorporation of the cell matrix construct into meniscus defects of rabbits after prior culturing in vitro possible, good regeneration of the meniscus defect, no proneness to inflammation in the knee joint.

5

In combination with the hyaluronic acid derivative, the adverse swelling in the case of gelatin or collagen otherwise occurring after moistening of the dry matrix is not produced. This is particularly favorable for tissue engineering matrices, in which a certain prespecified defect area is to be repaired.

10

The attached figures show magnifications of the composite matrix according to the invention:

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Figure 1 shows a 50 times magnification of a more stable structure (above) and a weaker structure (below),

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Figure 2 shows a 100 times magnification of the more stable structure (above) and the weaker structure (below),

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Figure 3 shows a 200 times magnification of the more stable structure (above) and the weaker structure (below) and

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Figure 4 shows chondrocytes growing in the pores under 200 times magnification (above) and 1000 times magnification (below).

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The more stable structure in each case in figures 1-3 was obtained by means of a higher total concentration of the matrix components in the solution during production, the total concentration being varied from 6.3% for the weaker structure to 9.2% for the more stable structure.

The threadlike structures in figure 4 indicate the beginning of production of the extracellular matrix.

The following examples are intended to explain the present invention in greater detail.

Example 1

A composite matrix according to the invention was produced by dissolution of HYAFF and gelatin in HFIP, addition of sodium chloride crystals, shaping and drying of the resulting paste, and by removal of the sodium chloride by repeated rinsing with water. After drying, the composite matrix was subjected to gamma sterilization.

Example 2

Description of production

Obtainment of chondrocytes for the primary cell culture

Adult joint cartilage/embryonic cartilage

After removal, hyaline joint cartilage (adult/embryonic) was immediately transferred to RPMI medium and worked up as rapidly as possible (less than 24 h). For this, the cartilage was first separated mechanically from the bone and then comminuted using a scalpel (final size: pieces of cartilage 1-2 mm in size). Enzymatic partial digestion with collagenase, hyaluronidase and DNase was then carried out at 37°C with continuous swirling. The enzymes were resuspended in RPMI medium with HEPES buffer, L-glutamine and addition of penicillin/streptomycin (antibiotic addition). The optimized enzymatic dissociation interval was 12 hours. The enzymatic activity was buffered by addition of serum-containing medium (RPMI with 10% AB serum or RPMI with 10% fetal bovine serum

(FBS). Pieces of extracellular matrix still remaining were then extracted by filtering and centrifuging the suspension, discarding the supernatant and resuspending the cell pellet in RPMI containing 10% AB serum or RPMI containing 10% FBS. After cell counting, stationary culturing and amplification of the chondrocytes for 14-21 days (2 times per week change of medium with serum-containing medium). After reaching confluence, the cells were passaged (removal of the medium, addition of 0.25% trypsin, after abolishment of cell adherence addition of serum-containing medium, centrifugation of the cell suspension and resuspension of the pellet then obtained in fresh medium, cell counting and renewed sowing of the cells). A 1 in 4 division was usually carried out here, i.e. one culture bottle yielded the cells for 4 new bottles. After renewed confluence (about 2-4 weeks), the cells (secondary culture) were detached from the culture bottom by renewed trypsin treatment (see above) and prepared for the treatment of the matrices.

It is also possible, however, to use cells in primary and tertiary culture (2 passaging steps).

Embryonic mesenchymal stem and progenitor cells from the umbilical cord

The cells were cultured in DMEM medium containing 10% FBS and, after reaching confluence, used as a primary culture for matrix loading. The adherent cells were again obtained by the application of trypsin described above.

Adult mesenchymal stem and progenitor cells from bone marrow

Bone marrow was obtained from the iliac crest of white New Zealand hares 4 months old. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum

(FBS) was added to the aspirate. After determination of the cell count, 20×10^6 cells were cultured at 37°C with 5% CO₂ in 100 mm culture dishes. The medium was changed twice per week until the cells were 80% confluent. Adherent cells were treated with trypsin as described above, counted, washed and resuspended in DMEM to a final concentration of 5×10^5 cells/25 µl.

Matrix treatment

10

For matrix treatment, the cell pellet was taken up in a little medium (1 mm³/1 µl). After this, the matrix (sterile, previously dry) was loaded from one side. The escape of air which was situated in the matrix was thereby ensured (time of action 1-5 minutes). After this, still remaining medium with a high cell concentration was transferred to the matrix by production of an under- and overpressure using a pipette tip. A distribution of cells in the matrix which is as homogeneous as possible should be achieved. The loading can be facilitated by the addition of detergents.

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A 2 h incubation in an incubator (37°C, 5% CO₂) then followed. This allowed the cells to adhere to the matrix present.

Finally, the cell-matrix construct was completely coated with medium and cultured further.

30

Description of culture

Stationary culture

35

For this, different culture media were used:

RPMI containing 10% AB serum (human),

RPMI containing 10% FBS or

Dulbecco's Modified Eagle's Medium (DMEM) having a high glucose content and nutrients and additives (ITS and pyruvate plus dexamethasone, ascorbic acid and TGF betal). (Cf. B. Johnstone in Exp. Cell Res. 238 (1998)).

Continuous perfusion culture

10 An alternative culturing condition is the continuous perfusion culture in RPMI medium containing 10% AB serum.

Example 3

15 In order to investigate the efficiency of reloading of the composite matrix according to the invention with cells, a composite matrix produced according to Example 1 was loaded with the bone marrow cells obtained
20 according to Example 2. Some of the matrices were immediately fixed in formalin, washed and embedded in paraffin (Group I). Other loaded matrices (Group II) were cultured for 14 days in a chondrogenic medium comprising DMEM with ITS + premixture (Collaborative
25 Biomechanical Products), pyruvate (1mM), ascorbic acid 2-phosphate (37.5 µg/ml), dexamethasone (10^{-7} M) and TGF-β1 (10 ng/ml). The medium was changed three times weekly.

30 The cell suspension was introduced into the matrix with slight swelling of the construct. By means of Toluidine Blue-stained areas of the matrices from Group I, it was possible to show that a high cell loading was present in all pores of the matrices. After 14 days under
35 chondrogenic culture conditions (Group II), the originally soft cell matrix constructs had hardened. The Toluidine Blue-stained areas showed cells having a marked metachromatically staining extracellular matrix,

which was present in the entire composite matrix. The extracellular matrix contained collagen II.

5

Example 4

Composite matrices loaded with cells as in Example 3 were implanted subcutaneously into immunodeficient mice (Group III). The same matrices were cultured for 14 days in vitro in the chondrogenic medium described in Example 3 and then implanted in vivo (Group IV). The implants were removed after 3 weeks, fixed in formalin, decalcified and embedded in paraffin.

15 5 μ m thick disks of the samples were cut and stained using Toluidine Blue. The stained areas were assessed according to their osteochondral differentiation (0 (neither bone nor cartilage in the matrix pores) to 4 (more than 75% of the pores contain bone and/or
20 cartilage)).

After 3 weeks in vivo, a significant size change occurred neither in the Group III nor in the Group IV implants. The implants precultured for 14 days in vitro
25 (Group IV) appear, however, to be qualitatively harder than the composite matrices which were implanted immediately after loading with the cells (Group III). The staining with Toluidine Blue produced an osteochondral differentiation of cells in composite
30 matrices of both groups, in which the pores filled with cartilage and bone. The composite matrices of Group IV, however, contained more bone and cartilage (average assessment = 4, compared with an assessment of 3 for Group III). Additionally, the content of bone in the
35 samples of Group IV was greater (ratio of bone: cartilage: fibrous tissue in Group III: 40:20:40 and in Group IV: 85:10:5).

Patent claims

1. A porous composite matrix, in which the matrix is constructed from matrix formers comprising a hyaluronic acid derivative and a hydrolyzed collagen, and the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 30:70 to 99:1.
2. The composite matrix as claimed in claim 1, in which the matrix formers are present in a weight ratio range of hyaluronic acid derivative to hydrolyzed collagen of 60:40 to 99:1, preferably in a weight ratio of approximately 70:30.
3. A composite matrix as claimed in one of the preceding claims, in which the hydrolyzed collagen is partially and/or completely hydrolyzed.
4. A composite matrix as claimed in one of the preceding claims, in which the hydrolyzed collagen is additionally derivatized and/or crosslinked.
5. A composite matrix as claimed in one of the preceding claims, in which the hyaluronic acid derivative is a hyaluronic acid ester.
6. A composite matrix as claimed in claim 5, in which the hyaluronic acid ester is an ethyl or benzyl ester of hyaluronic acid.
7. A composite matrix as claimed in one of the preceding claims, comprising pores having an average diameter in the range of 10-1000 μm .
8. A composite matrix as claimed in claim 7, in which the pores have an average diameter in the range of 100-350 μm .

9. A composite matrix as claimed in claim 7, in which the pores have an average diameter in the range of 350-1000 μm .
- 5 10. A composite matrix as claimed in claim 8 or 9, in which pores in the range of 10-100 μm are additionally present.
- 10 11. A composite matrix as claimed in one of the preceding claims, which has crosslinkages.
- 15 12. A composite matrix as claimed in one of the preceding claims, comprising biologically active compounds such as antibiotics, compounds for improving cell adhesion, calcium salts, inductive factors or further glycosaminoglycans and their derivatives.
- 20 13. A composite matrix as claimed in one of the preceding claims, comprising chondrocytes, mesenchymal stem and progenitor cells, osteoblasts or connective tissue cells.
- 25 14. A process for the production of a porous composite matrix as claimed in one of claims 1-13, comprising the dissolution or suspension of the hyaluronic acid derivative and the hydrolyzed collagen in a suitable first solvent, the addition of a pulverulent compound which is virtually insoluble in the first solvent, but which is soluble in a second solvent, in which the matrix formers hyaluronic acid derivative and hydrolyzed collagen are virtually insoluble, to the solution or suspension, the pulverulent compound having an average particle size distribution in the range of the desired pore size of the composite matrix to be produced, the removal of the first solvent and subsequently the dissolution of the pulverulent compound in a second solvent, in which the
- 30
- 35

pulverulent compound dissolves and the matrix formers are virtually insoluble.

15. The process as claimed in claim 14, in which the first solvent is 1,1,1,3,3,3-hexafluoro-isopropanol.
16. The process as claimed in claim 14 or 15, in which the pulverulent compound is a water-soluble alkali metal or alkaline earth metal salt, in particular an alkali metal halide such as sodium chloride.
17. The process as claimed in one of claims 14-16, in which the second solvent is water.
18. The process as claimed in one of claims 14-17, in which the composite matrix is additionally shaped, dried and optionally sterilized.
19. The process as claimed in one of claims 14-18, in which the composite matrix is additionally optionally loaded with biologically active compounds and chondrocytes, mesenchymal stem and progenitor cells, osteoblasts or connective tissue cells.
20. The use of a composite matrix as claimed in one of claims 1-13 for the generation of differentiated tissue from chondrocytic cells or mesenchymal stem and progenitor cells, freshly removed or amplified cells being added to the composite matrix and optionally cultured under chondro-, osteo- or fibrogenic conditions.
21. The use as claimed in claim 20 for the tissue engineering of tissue types of the connective and supportive apparatus, in particular of chondral and osseous tissue.

22. The use as claimed in claim 20 for the in-vivo differentiation of the cells to tissue types of the connective and supportive apparatus, in particular to chondral and osseous tissue.

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23. An implant, comprising a porous composite matrix as claimed in one of claims 1-13.

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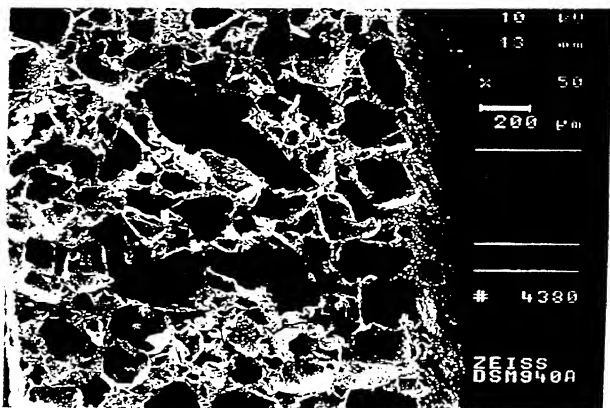
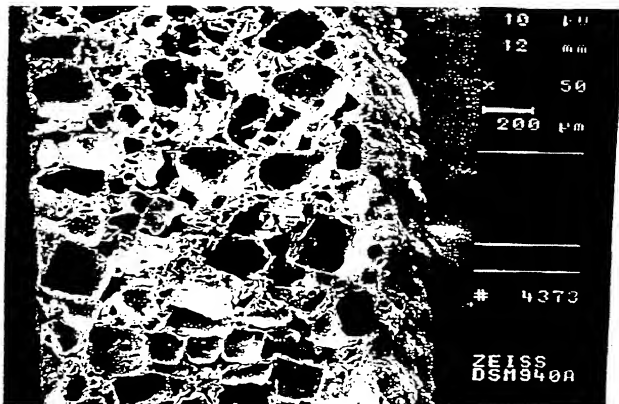
24. A process for the production of an implant as claimed in claim 23, in which a porous composite matrix as claimed in one of claims 1-13 is coated onto the implant surface.

Abstract

The invention relates to a porous composite matrix formed from a hyaluronic acid derivative and hydrolyzed collagen, which is used as a biocompatible and biodegradable composite matrix for the tissue engineering of chondral and osseous tissue and for the repair of musculoskeletal defects.

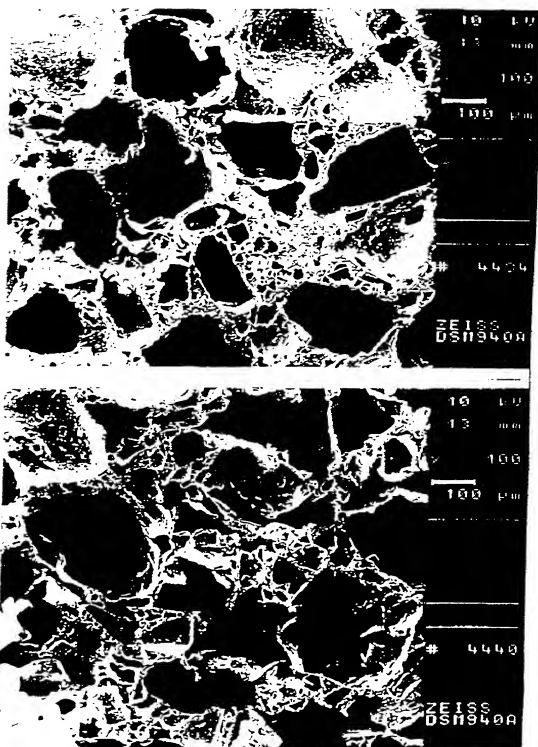
Figure 1

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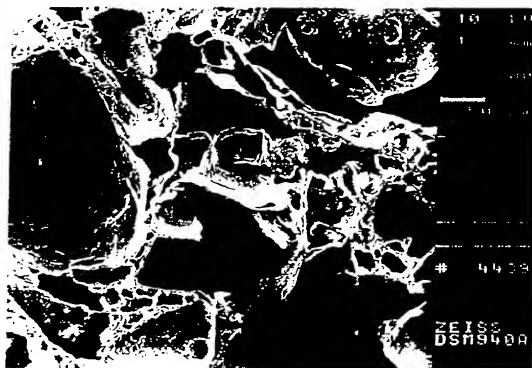
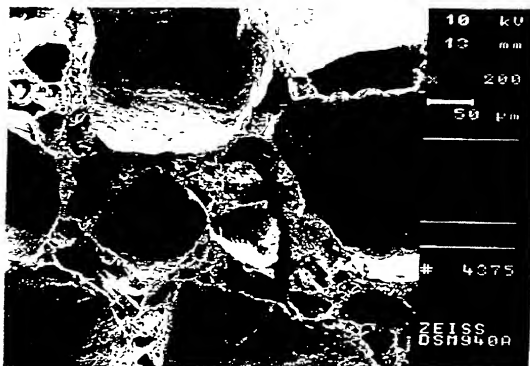
Figure 2



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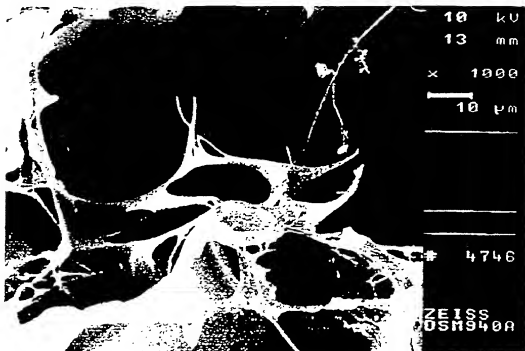
Figure 3

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DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

POROUS COMPOSITE MATRIX AND THE PRODUCTION AND USE THEREOF

the specification of which (check one)

☐ is attached hereto.

☒ was filed on December 3, 1999 as U.S. Serial No. _____ or PCT International Application Number PCT/EP99/09444 and was amended on _____

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

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Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?
198 55 890.2	Germany	December 3, 1998	Yes	No

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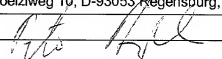
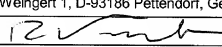
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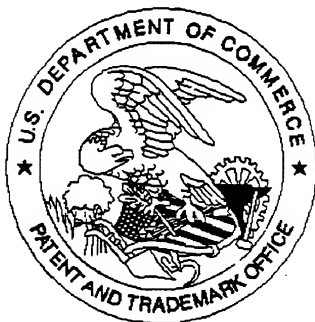
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